Prior to addressing the rejections based on the prior art, Applicant would like to first discuss various statements made by the Examiner in the last Official Action in order to clarify the record. More specifically, the Examiner "contends that the specification is silent with respect to M-tropic strains, as well as the fact that "the arguments filed in the last response were not commensurate in scope with the claims as instantly recited."

The Applicant respectfully disagrees with the above conclusions of the Examiner for the following reasons.

Applicant submits that it is well known in the art that M-Tropic HIV strains means macrophage-tropic strains. See, for instance, Annex I wherein macrophage-tropic and M-tropic are used interchangeably.

Moreover, it is clearly explained in the translation in Annex 2 that at the early onset of contamination of HIV, the virus has more particularly a tropism for macrophages and due to this tropism, the virus infects in a privileged manner monocytes and macrophage cells.

It should be recalled at this point that monocytes are cell precursors of macrophages and monocytes become macrophages in tissues. Therefore M-Tropic strains of HIV are those strains that are derived from the monocyte/macrophage lineage (See, Annex 3).

The Examiner's contention that the specification is silent with respect to M-Tropic strains is unfounded. The specification at least at the paragraph bridging pages 1 and 2 states the following:

> Furthermore, the inhibition of a virus-producing infection in the monocytes appears to be linked to a large extent to the inhibition of the monocytic proliferation, which suggests that the replication of the virus depends on a preliminary obligatory stage of high proliferation of the monocyte cell. Thus, the proliferation of this population is thought to be an obligatory passage for the manifestation of the infectious HIV character. Thus, the hypothesis has been formulated that substances capable of inhibiting monocyte replication might also inhibit the

replication of HIV (J. Clinical Investigation, Vol. 89, pages 1154-1160, 1992).

The above paragraph, set forth in the background of the present specification, clearly introduces what the inventor has later proven and described in the rest of the specification; i.e., that complete inhibition of the replication of HIV in primary cultures of human monocytes is achieved with various muramyl peptides.

Indeed, at least page 6 of the specification at lines 28 to 38, it is explained that in Example 2 primary cultures of human monocytes were collected from healthy volunteers and that these cultures were infected "on day 0 with an HIV source (HTLV-III Ba-L) which exhibits a tropism for the monocytes." Different concentrations of the muramyl peptides were added at various time frames and HIV replication was measured. Table 2 shows the results achieved.

Thus the specification clearly describes inhibition of HIV in primary cultures of monocytes which are M-Tropic HIV strains.

Moreover many of the claims recite that the effective amount of the muramyl peptide is "an amount that is capable of causing 100% inhibition of the replication of the retroviruses in primary cultures of monocytes of the host." Thus, Applicant submits that many of the claims are commensurate in scope with the argument.

Furthermore, the specification does not teach the use of T-Tropic HIV strains which are lymphocyte tropic strains. Hence, the person skilled in the art would realize that the invention pertains to M-Tropic strains both as claimed and as described.

Finally the Examiner further contends that since treatment may be needed at any stage of HIV-1 infection that it is not beneficial to find drugs that target the early stage of HIV-1 infection. Applicant totally disagrees with the Examiner's reasoning. Indeed, if HIV-1 was targeted at an early stage when the patient is asymptomatic, there would be no need to diagnose and treat those patients that are symptomatic which have various disorders associated with AIDS such as Kaposi's sarcoma, *Pneumocystis carinii, cytomegalovirus, Candida albicans, Mycobacterium avium*

intracellulaire and the like, to mention a few. Thus, the costs for diagnosis and medical treatment required after a patient has symptomatic AIDS is considerable. Targeting the patient when asymptomatic clearly would reduce medical costs.

Turning now to the Official Action, Claims 14 to 21, 25, 26, 28 to 30 and 34 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Schreck et al. This rejection is respectfully traversed.

Schreck et al describe various muramyl peptides that were tested for NF-κB activity for possible use as adjuvants in AIDS vaccines. Basically the experiments set forth in Schreck et al display the possibility of using an adjuvant which would not stimulate NF-κB activity, since, as pointed out at page 188, first column under "Introduction":

(1) In monocytic cell lines bearing HIV-1 provirus, viral production is stimulated by agents that induce activation of NF-κB.

(2) The possibility that NF-κB may even be responsible for maintaining HIV-1 replication.

(3) Thus, in view of (1) and (2) one should avoid the use of adjuvant components in an AIDS vaccine that should be administered prophylactically at a population level or therapeutically to a seropositive individual that stimulate NF-κB.

More specifically, in the materials and methods section of Schreck et al it is clear that **there is no use of HIV-1 infected cells** in the experiments. In the reagents section, various muramyl peptides, as well as other reagents were disclosed. The cells lines that were used were **non-HIV infected** human Jarkat T cells, **non-HIV infected** human monocyte-macrophage cell lines and the **non-HIV infected** mouse pre-B cell line 70Z/3.12 (ATCC No. TIB 158), as well as various growth mediums.

In contrast in the present specification in the Examples, HIV-infected primary cultures of monocytes are used to test the inhibitory action of the muramyl peptides.

Thus, there is no suggestion nor any scientific evidence presented in Schreck et al that the various muramyl peptides disclosed therein can inhibit the replication of

acquired immunodeficiency viruses by administering an amount effective that is capable of causing 100% inhibition of said retroviruses in primary cultures of monocytes of the host.

Schreck et al never demonstrated that muramyl peptides had the capacity to inhibit NF-κB activation which might have led the skilled artisan to believe that the muramyl peptides may suppress viral replication in infected cells. Thus, there is no indication in Schreck et al that muramyl peptides alone can inhibit HIV replication or inhibit cellular pathways such as NF-κB activation that the skilled person might ascertain as possibly leading to viral suppression.

Moreover, it also appears that the Examiner relies on a nonexperimentally demonstrated sole statement in Scheck et al that "muramyl peptides are among candidate **adjuvants** that can be used in experimental AIDS **vaccines**" in maintaining this rejection.

But an adjuvant used in the context with a vaccine is only a nonspecific generic stimulator of the immune response added to a vaccine to improve the immune response so that less vaccine is needed. The antigen(s) in the vaccine generate(s) the needed antibody response.

Indeed, the one skilled in the art would appreciate that an adjuvant is a substance which assists to hasten or increase the action of a principal ingredient. This definition is clearly set forth in Annex 4, which specifically states the following:

> adjuvant 1. That which assists, esp. a drug added to a prescription **to hasten or increase the action of a principal ingredient** (emphasis added).

Therefore an adjuvant, by definition and appreciated by those skilled in the art, is **never a principal ingredient**.

Applicant submits that the use of the true definitions of the claims in the present response compared to the teachings of the prior art is not merely an

argument based on semantics. Rather, the skilled artisan is quite aware of the scientific purpose of an adjuvant used in conjunction with a vaccine.

A vaccine requires the use of the relevant protective antigen(s) to evoke an antibody response such that active immunological prophylaxis is achieved (see, Annex 5). In a vaccine the antigen(s) is the principal ingredient, even when used with an adjuvant. In the case of an AIDS vaccine, the protective HIV-1 antigens are required. However, none of these antigen(s) are described or even suggested in Schreck et al. Therefore Schreck et al do not teach anything about inhibiting HIV-1 using muramyl peptides.

Thus, the Examiner's conclusion that the presently claimed invention reciting a process to inhibit the replication of acquired immunodeficiency retroviruses by the claimed muramyl peptides wherein 100% inhibition is achieved in primary cultures of monocytes of the host is anticipated by Schreck et al cannot be maintained.

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 14 to 21, 25, 26, 28 to 30 and 34 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Masihi et al. This rejection is respectfully traversed.

It should be clear on the record that the teachings that MDP (muramyl dipeptide) inhibits immunodeficiency virus *in vitro* as described by Masihi et al, does not encompass any of the claims of the present invention. The claims of the present invention do not recite or include MDP as a compound. This is evidenced in Annex 6 wherein, when compared to the presently claimed formula, R1 = NH_2 and R2=OH for MDP. In contrast in the presently claimed invention R1=O(CH2)x where x=1,2,3, or 4 and R2=an amino or O(CH2)xH. Therefore MDP is not being claimed.

Indeed, Masihi et al teach a weak inhibition of T-Tropic HIV strains in various cell lines such as U937, H9 and KE37/1 with MDP. This is apparent under the Discussion section at page 395. Furthermore, it is clear that Masihi et al disclose that

the effect of MDP on normal human monocytes infected with HIV have not been tested (See, page 397, first full paragraph, last sentence).

It appears that the Examiner has neglected the teachings of Masihi et al as a whole in rendering this rejection and relies only through hindsight on one passage of Masihi et al that states the following:

> A nonpyrogenic butyl ester analog of MDP, murabutide has been used as an adjuvant in human clinical trials.

As stated above, an adjuvant is a substance added to a vaccine to improve the immune response so that less vaccine is needed. Antigen(s) are the principal ingredients of a vaccine. Without antigen(s) the vaccine will not produce the required antibodies needed for immunological prophylaxis.

Indeed, in the results section of Masihi et al MDP that was used to inhibit T-Tropic HIV replication in cell lines was never mentioned as an adjuvant or having adjuvant activity. Rather, MDP was mentioned alone in its modulation of T-Tropic HIV strains; i.e., as the principal ingredient.

The Examiner purports that an adjuvant is a principal ingredient, which means "nothing more than a most important element." However, as demonstrated above, an adjuvant is not a principal ingredient as confirmed in Annex 4. Moreover, a person skilled in the art would realize that the term "principal ingredient" does not refer to the quantity of substance in a drug, but that ingredient which is the most active substance. In a vaccine, administered with an adjuvant, the principal ingredient is the antigen(s). Without the antigen(s) there would be no immunological prophylaxis and hence the drug would not work.

Since Masihi et al is silent with teaching muramyl peptides that are currently claimed as inhibiting HIV in primary cultures of monocytes, it cannot be said that this reference anticipates the claimed invention.

Thus, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 14 to 21, 25, 26, 28 to 30 and 34 have been rejected under 35 U.S.C. § 103 (a) as being unpatentable over by Masihi et al. This rejection is respectfully traversed.

Masihi et al was discussed in detail above with respect to novelty and the same arguments are incorporated herein by reference. As set forth above this reference discloses MDP as the principal ingredient to inhibit T-Tropic HIV-1 replication in cell lines. There is no suggestion in Masihi et al to use different muramyl peptides in a process to inhibit HIV-1 in M-Tropic strains ; i.e., primary cultures of monocytes.

Furthermore, as explained above, an adjuvant is not a principal ingredient (See, Annex 4).

Moreover, it has been well known since over a decade that M-Tropic and T-Tropic strains use different pathways to infect cells and that T-Tropic strains cannot infect macrophages. It is also known that molecules which inhibit infection with T-Tropic strains do not necessarily inhibit infection with M-Tropic strains of HIV-1. More importantly, the various steps involved in the in the establishment of infection and in the completion of the virus life cycle are known to be different between macrophages, T lymphocytes and cell lines. All this has been substantiated by the findings that M-Tropic HIV-1 strains use a coreceptor on macrophages that is different from the coreceptor used by T-Tropic strains to infect cell lines. Thus, the teachings of Masihi et al only demonstrate that MDP can weakly reduce T-Tropic HIV replication in cell lines. These results do not encourage, but would have discouraged, a skilled artisan to predict that other muramyl peptides such as Murabutide will have the capacity to inhibit up to 100% the replication of M-Tropic HIV strains in primary monocyte/macrophage cells. Therefore, the findings and the claims of the present invention could not have been predicted, let alone obvious, from the reported results of Masihi et al.

Finally, it should be noted that in the present application 100% inhibition of human immunodeficiency virus was obtained using primary cultures of monocytes. In contrast in Masihi et al only 47% inhibition was achieved using T-Tropic cell lines. Thus it is still an unexpected result that total inhibition was achieved using the claimed muramyl peptides, while the prior art taught that only 47% could be achieved in the strains used.

Thus, in view of the above, withdrawal of this rejection is respectfully requested.

From the foregoing, favorable action in the form of a Notice of Allowance is respectfully requested and such action is earnestly solicited.

OIPE JC68
MAR 12 2001
PATENT & TRADEMARK OFFICE

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, Ph.D. (Reg. No. 40,069) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee of $445.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By MaryAnne Armstrong (Reg No. 40,069)
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ANNEX I

MeSH Heading (Major)
HIV-1|*PH; RANTES|*PH; Tropism|*; Virus Replication|*PH
MeSH Heading
Down-Regulation (Physiology); Human; Macrophage Inflammatory Proteins|PH; Receptors, CCR5|GE; Virus Inhibitors

Publication Type
MEETING ABSTRACTS
Country of Publication
UNITED STATES

Record 2 from database: **AIDSLINE**

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Title
Expression of CD4 and chemokine receptors in human brain cells: astrocyte entry by HIV-1 T and M tropic strains is blocked by SDF-1 and RANTES.
Author
Taoufik Y; Boutet A; Lannuzel A; Salim H; Azzarone B; Dussaix E; Vincent JD; Tardieu M; Delfraissy JF
Address
Laboratoires Virus, Neurones et Immunite, France.
Source
Conf Retroviruses Opportunistic Infect, 1998 Feb, 5th:, 163 (abstract no. 448)
Abstract
HIV-1 entry into target cells requires at least two key molecules at the cell surface, namely the CD4 receptor and specific chemokine receptors. Among the latter, CCR-5 and CXCR-4 permit the entry of macrophage-tropic (M) and T-cell line-adapted (T) strains, respectively. HIV-1 infects the central nervous system (CNS) and causes severe neurological manifestations, particularly the AIDS dementia complex syndrome (ADC). However, the precise target cells in the central nervous system and the mechanisms of viral entry remain to be identified. Here, we report that 1) the CD4 receptor is expressed by astrocytes, microglial cells and neurons; 2) beta-chemokine receptors and CXCR-4 are expressed as functional receptors by the three CNS cell types; 3) both M- and T-tropic HIV-1 strains can efficiently enter astrocytes, the most abundant cell type in the brain. 4) viral entry is inhibited by RANTES and SDF-1.
Language of Publication
English
Unique Identifier
98929379

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MeSH Heading (Major)

Antigens, CD4|*ME; Brain|CY/*VI; Chemokines|*PD; HIV-1|*PH; Receptors, CCR5|*ME; Receptors, CXCR4|*ME

MeSH Heading

Astrocytes|ME; Microglia|ME; Neurons|ME; RANTES|PD

Publication Type

MEETING ABSTRACTS

Country of Publication

UNITED STATES

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ANNEX

Tome 2

14e édition

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Factors that Determine th Cellular Tropisms of HIV

At the beginning of the natural history of the illness the virus has a tropism for macrophages that predominates in the blood while gradually as the illness progresses it is the lymphocyte tropism that predominates. Likewise the viral organism isolated in patients at the beginning of the illness have a NSI phenotype in cell cultre (non-synctia inducible which means non-inducer of syncitium) for *in vitro* investigations. The isolated virus in advanced stages of the illness have preferably more of the phenotype SI (SI: synctia-inducible which means inducer of syncitium) (see below). Also the term macrophage tropism is frequently used for designating the expression of the phenotype NSI and the term Lymphocyte T tropic to designate the phenotypy SI. The passage in an infected individual that has a predominant of organisms having macrophage tropic/NSI to the predominance of organisms lymphocyte tropic/SI (that does not imply the transitory presenece of the conconmitant virsu expressing the two phenotypes) marks a rupture in the progression of the illness, with, as a consequence, a dimunition very rapidly of a number of lymphocytes T CD4+.

At the moment of contamination, the virus that infects has more particularly a tropism for macrophages. Neverthelesss, since the majority of studies that have been performed in male homosexuals infected during anal sex, this information is a bit biased by the nature of the particular mode of transmission. The viral organisms macrophage tropic infect in a privileged manner are the monocytes-macrophages and the microglial cells in the brain.

…té de la protéine d'enveloppe gp… du VIH-1, tout par-…ment dans la troisième région variable (boucle V3) (voir …rus). Au début de l'histoire naturelle de la maladie, les …tropisme macrophagique prédominent dans le sang tan… …n fur et à mesure que la maladie progresse, ce sont les …cytotrop qui prédominent. De même, les souches virales … chez des patients au début de leur maladie ont un phé… NSI en culture cellulaire (NSI : *non-syncytia-inducible* …non inducteur de syncitium) selon des examens *in vitro*. …ns isolés à un stade avancé de la maladie ont plus vo… un phénotype SI (SI : *syncytia-inducible* c.-à-d. in… de syncitium) (voir ci-dessous). Ainsi, le terme *à tro… macrophagique* est souvent utilisé pour désigner l'ex… …on phénotypique *NSI* et le terme *lymphocyte T tropique*, …désigner le phénotype *SI*. Le passage, chez un individu in… d'une prédominance de souche à tropisme macropha… …NSI à une prédominance de souches lymphocytotropes/SI …i peut impliquer transitoirement la présence concomitante …s exprimant les 2 phénotypes), marque une rupture dans …gression de la maladie, avec, par la suite, une diminution …pide du nombre de lymphocytes T CD4+.

…moment de la contamination, les virus infectants sont plu… …tropisme macrophagique. Cependant, comme la plupart de …udes ont été réalisées chez des homosexuels masculins in… au cours de rapports anaux réceptifs, cette information est …être biaisée par la nature particulière de cette voie de trans… …ion. Les souches virales macrophages tropiques infectent de … privilégiée les monocytes-macrophages et les cellules mi… …liales du cerveau. La molécule *galactosylcéramide* peut …stituer un récepteur de rechange pour le VIH dans les cel… …neuronales et épithéliales n'exprimant pas le marqueur …(voir la section sur la neuropathogénie ci-dessous). Le gène …est principalement responsable des différences dans la ca… …lité des souches virales à infecter certains types cellulaires …oculiers, par exemple, des lymphocytes plutôt que des mo… …tes, et des cellules mononucléées primitives plutôt que des …ules de lignées cellulaires. La région V3 de la protéine d'en… …oppe virale est déterminante pour le tropisme cellulaire et …ion rôle dans la fusion du virus et de la membrane cellu… …. Certaines parties du gène codant la protéine d'enveloppe, …ées en dehors de la région V3, peuvent également influen… le tropisme cellulaire du VIH; cependant, les mécanismes en …ue n'ont pas encore été élucidés.

Au cours de l'année 1996, on a montré que le tropisme cel… …ne rendait en fait compte de la présence de corécepteurs du …H-1 et qu'il pouvait être spécifique, soit des souches à tro… …isme macrophagique, soit des souches lymphocytotropes. Ces …récepteurs font partie de la famille de récepteurs couplés à la …V protéine G à 7 domaines transmembranaires. Le corécepteur des souches lymphocytotropes est la *fusine* ou CXCR4, qui est indispensable à la fusion et à l'entrée de ces souches virales dans la cellule. Son ligand naturel est le SDF-1 *(stromal-cell—derived factor)*. Ce ligand et des anticorps anti-fusine peuvent bloquer efficacement l'entrée des souches virales lymphocytotropes et l'infection des cellules cibles. Le principal corécepteur pour les virus à tropisme macrophagique est le *CCR5*, récepteur de chimiokines β. Ce récepteur lie les 3 chimiokines β (RANTES, MIP-1 α, MIP-1 β) dont on a montré la capacité à inhiber l'infection cellulaire par les souches à tropisme macrophagique. Cependant, il y a beaucoup de redondance parmi les récepteurs de chimiokines β et d'autres membres de cette famille, en particulier CCR2b et CCR3, peuvent faciliter l'infection cellulaire par des souches de VIH-1 à double tropisme macrophagique et lymphocytaire. Les mécanismes d'inhibition de l'entrée des souches à tropisme macrophagique et lymphocytotropes de VIH dans leurs cellules cibles par les chimiokines β et par SDF-1 respectivement, ne sont pas parfaitement clairs. Cependant, certaines expériences montrent que, au moins pour les chimiokines β, le mécanisme passe par un blocage des récepteurs, et non par une inhibition de la transduction du signal (figure 308-16). Du fait de l'importante complexité de l'interaction entre le VIH et son hôte humain, il est très vraisemblable que d'autres corécepteurs de l'entrée du VIH-1 seront identifiés dans le futur.

ANOMALIES DES LYMPHOCYTES T Les anomalies des cellules T à un stade avancé de l'infection à VIH sont variées. Elles sont à la fois quantitatives et qualitatives, et touchent toutes les branches du système immunitaire (voir ci-dessous), montrant à quel point l'intégrité du système immunitaire dépend des fonctions inductrices/auxiliaires des lymphocytes T CD4+. Presque tous les déficits immunitaires observés à un stade avancé de l'infection par le VIH peuvent finalement s'expliquer par le déficit quantitatif en lymphocytes T CD4+. Cependant, des anomalies fonctionnelles portant sur les lymphocytes T (voir ci-dessous) peuvent être mises en évidence précocement au cours de l'évolution de l'infection, même lorsque le taux de lymphocytes T CD4 demeure dans des valeurs normales. L'intensité et la variété des anomalies augmentent au fur et à mesure que l'infection progresse. L'une des premières anomalies détectables rend compte d'un défaut de réponse aux antigènes de rappel comme la toxine tétanique ou le virus de la grippe, à un moment où les cellules mononucléées sont toujours capables de répondre normalement à une stimulation mitogène. Aux altérations de la réponse aux antigènes solubles fait suite la perte de la réponse proliférativ

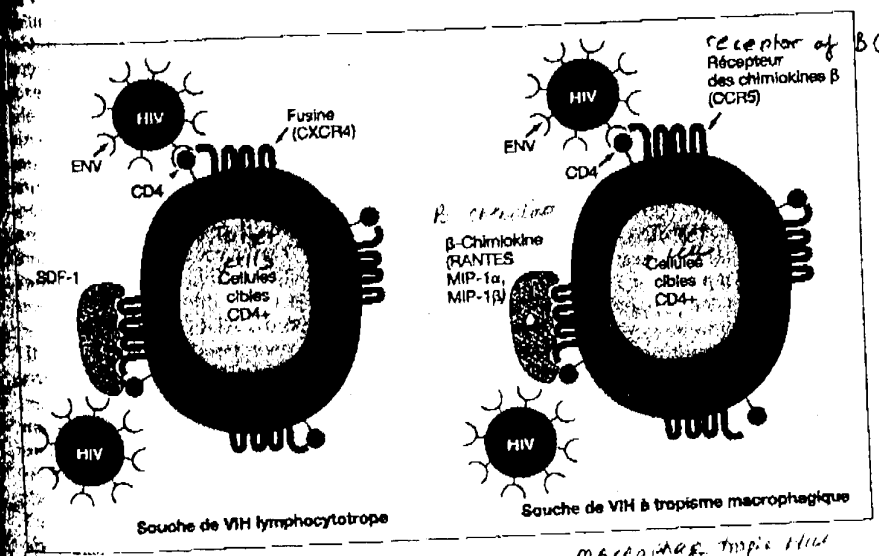

Figure 308-16 Modélisation c… rôle des corécepteurs (fusine … CCR5) dans la liaison et l'entrée d… souches de VIH lymphocytotrope… et à tropisme macrophagique, da… leurs cellules cibles CD4+ respe… tives. Le blocage de cet événeme… très précoce du cycle cellulaire vi… peut être réalisé par l'inhibition … la liaison aux corécepteurs par le… gand du récepteur en question. … ligand de la fusine est le SDF… *(stromal-cell—derived factor)*; les … gands pour le récepteur de chim… kines β CCR5 sont RANTES, MIP… et MIP-1β.

IN THE UNITED STATES PATENT OFFICE

I, Julia Andral-Ziurys, declare the following:

1. That I am a resident of Villennes sur Seine, France.
2. That I am well acquainted with the French and English languages.
3. That the attached translation of Harrison's Internal Medicine is, to the best of my knowledge and belief, a true translation into the English language of the accompanying document.

March 1, 2001
Date

Julia Andral-Ziurys

ANNEX 3

MOLECULAR BIOLOGY OF THE CELL

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

Garland Publishing, Inc.
New York & London

"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson
The Cell in Development and Heredity
3rd edition, 1925, Macmillan, Inc.

Bruce Alberts received his Ph.D. from Harvard University and is currently a Professor in the Department of Biophysics and Biochemistry at the University of California Medical School in San Francisco. *Dennis Bray* received his Ph.D. from the Massachusetts Institute of Technology and is currently a Senior Scientist in the Medical Research Council Cell Biophysics Unit at King's College London. *Julian Lewis* received his D.Phil. from Oxford University and is currently a Lecturer in the Anatomy Department at King's College London. *Martin Raff* received his M.D. degree from McGill University and is currently a Professor in the Zoology Department at University College London. *Keith Roberts* received his Ph.D. from Cambridge University and is currently Head of the Department of Cell Biology at the John Innes Institute, Norwich. *James D. Watson* received his Ph.D. from the University of Indiana and is currently Director of the Cold Spring Harbor Laboratory. He is the author of *Molecular Biology of the Gene* and, with Francis Crick and Maurice Wilkins, won the Nobel Prize in Medicine and Physiology in 1962.

Cover photograph kindly provided by Michael Verderame and Robert Pollack of Columbia University. The fluorescein-phalloidin used to stain the actin cables was the generous gift of Drs. Theodor Wieland and A. Deboben of the Max Planck Institute, West Germany. The photograph is of a mouse fibroblast that had been transformed to anchorage-independent growth by the virus Simian Virus 40 (SV40) and subsequently selected for anchorage-dependent growth. This particular cell was stained for SV40 large T antigen (*red*) and fluorescein-phalloidin (*green*), which specifically stains F actin.

© 1983 by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson.

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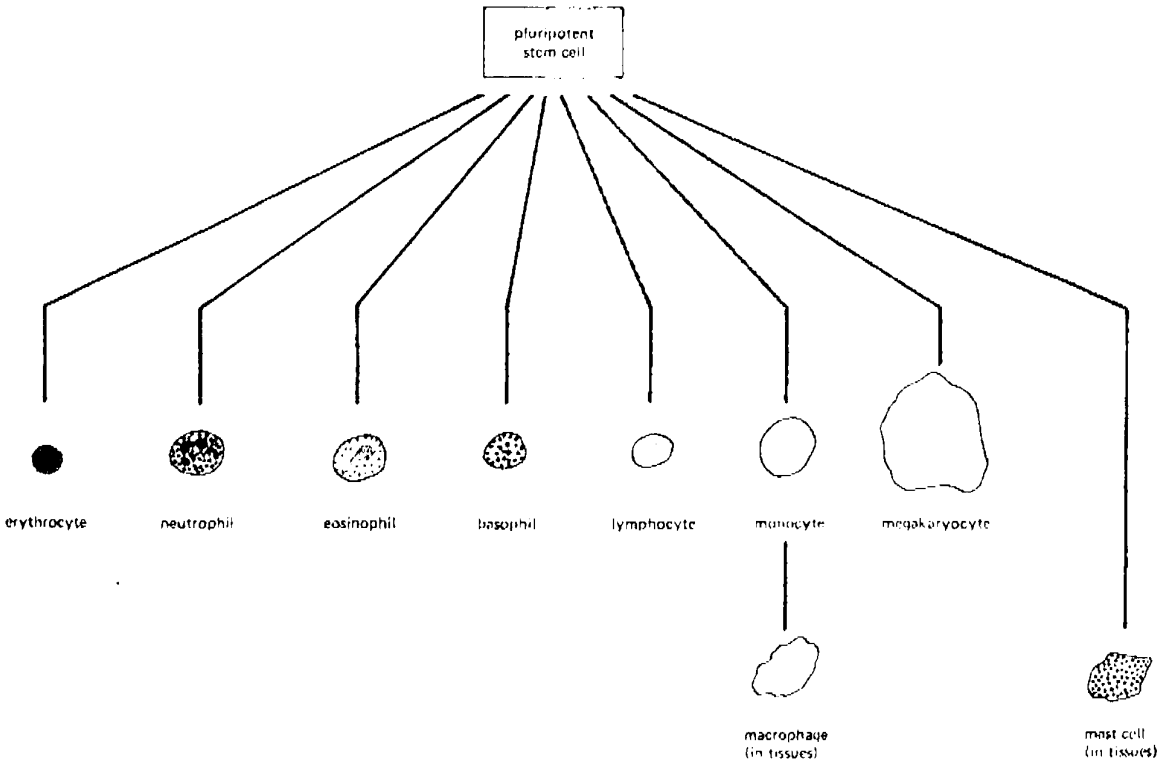

Figure 16–38 The different classes of cells that derive from the pluripotent hematopoietic stem cell.

between several alternative lines of differentiation. This choice might be made at random, or it might, for example, be controlled by the environment of the stem cells. Though there has been much debate, the problem of what governs the choice is still not resolved.

The Number of Specialized Blood Cells Is Amplified by Divisions That Follow Commitment[27]

Once a cell has differentiated as an erythrocyte or a granulocyte or some other type of blood cell, there is no going back: the state of differentiation is not reversible. Therefore, at some stage in their development, the progeny of the pluripotent stem cell must become irreversibly committed or determined for a particular line of differentiation. At what stage does this commitment occur? It is clear from simple microscopic examination of the bone marrow that it happens well before the final division in which the mature differentiated cell is formed: one can recognize specialized precursor cells that already show signs of having begun differentiation but are still proliferating. It thus appears that commitment to a particular line of differentiation is followed by a series of cell divisions that amplify the number of cells of a given specialized type. In this way, a very small number of pluripotent stem cells serve to generate very large numbers of differentiated blood cells. Furthermore, it turns out that the amplifying divisions are subject to important controls that regulate the production of each type of blood cell according to need. Such controls are especially well documented for the cell lineage committed to erythrocyte formation.

Figure 16–31 Scanning electron micrograph of mammalian blood cells in a small blood vessel. The larger, more spherical cells with a rough surface are white blood cells; the smaller, smoother flattened cells are red blood cells. (From R. G. Kessel and R. H. Kardon, Tissues and Organs: A Text-Atlas of Scanning Electron Microscopy. San Francisco: Freeman, 1979. © 1979 W. H. Freeman and Company.)

they take their individual names from the different staining properties of the granules. The differences of staining reflect major differences of chemistry and function. Neutrophils, the commonest type, engulf, kill, and digest bacteria. Lymphocytes comprise a functionally heterogeneous group of cells all concerned with immune responses; in addition, there are *killer cells* that look like lymphocytes and function as accessory cells in immune responses but are not part of the immune system proper. Monocytes, on leaving the bloodstream, become *macrophages*, which can dispose of invading microorganisms, foreign bodies, and cellular debris by phagocytosis. Neutrophils and macrophages are the main "professional phagocytes" in the body.

Table 16–1 Blood Cells

Type of Cell	Main Functions
Red blood cells (erythrocytes)	transport O_2 and CO_2
White blood cells (leucocytes)	
Granulocytes	destroy invading bacteria
Neutrophils (polymorphonuclear leucocytes)	
Eosinophils	destroy larger parasites and modulate allergic inflammatory reactions
Basophils	release histamine and serotonin in certain immune reactions
Lymphocytes	make immune responses
Killer cells	kill virally infected cells and some tumor cells
Monocytes	become macrophages in the tissues
Megakaryocytes, giving rise to *platelets*	initiate blood clotting

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adjuvant

1. That which assists, esp. a drug added to a prescription to hasten or increase the action of a principal ingredient. 2. In immunology, aluminum salts such as aluminum hydroxide and aluminum phosphate that are added to an antigen to increase the body's immunologic response. The adjuvants increase the size of the antigen, making it easier for B lymphocytes and phagocytes to recognize it, promote chemotaxis, and stimulate the release of cytokines. Adjuvants are not effective with all antigens and do not stimulate T lymphocyte activity.

Freund's complete adjuvant A water-in-oil emulsion in which an antigen solution is emulsified in mineral oil with killed mycobacteria to enhance antigenicity. The intense inflammatory response produced by this emulsion makes it unsuitable for use in humans.

Freund's incomplete adjuvant A water-in-oil emulsion in which an antigen solution without mycobacteria is emulsified in mineral oil. On injection, this mixture induces a strong persistent antibody formation.

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ANNEX 5

IMMUNOLOGY

Ivan Roitt · Jonathan Brostoff · David Male

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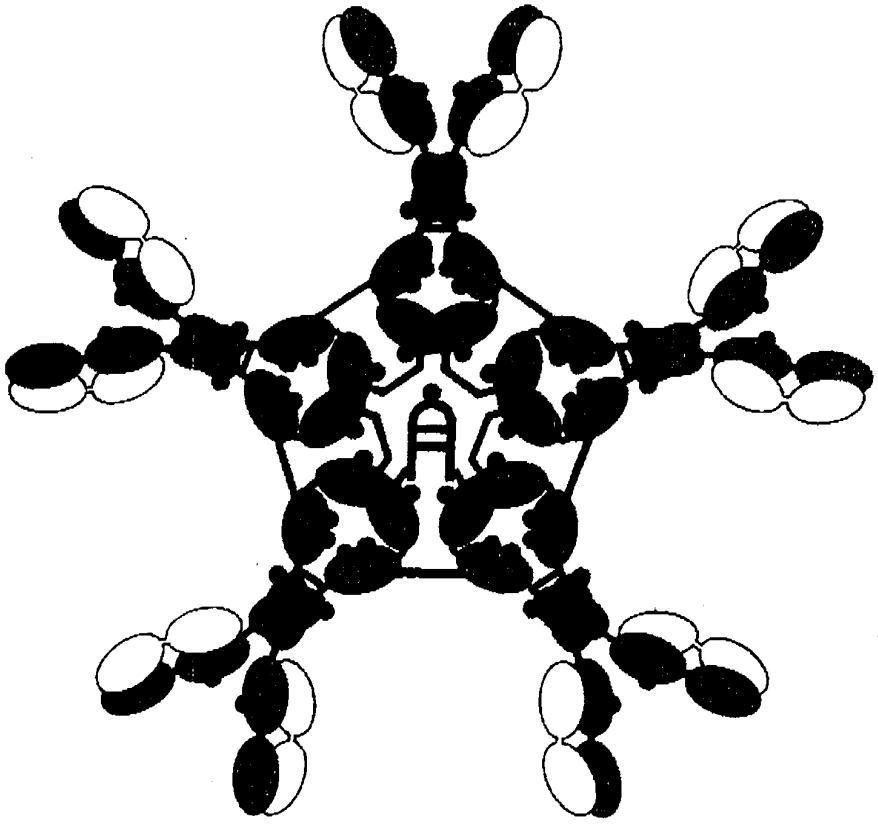

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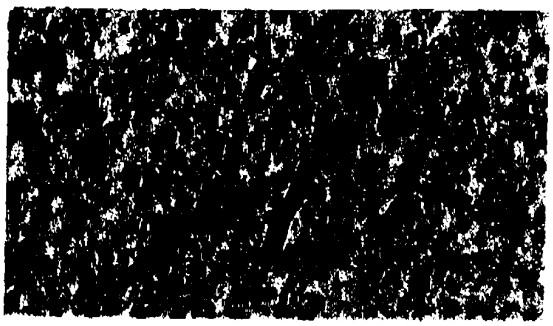


Fig. 16.30 Evidence for neutrophil-mediated immunity to mucormycosis. Section through a lung of a patient suffering from mucormycosis – an opportunistic infection in an immunosuppressed subject. The inflammatory reaction consists almost entirely of neutrophil polymorphs around the fungal hyphae. The disease is particularly associated with neutropenia. Silver stain, × 400. (Courtesy of Dr R.J. Hay.)

Candida albicans	+	(+)	(+)
Candida pseudotropicalis	+	–	+
Cryptococcus neoformans	+		+
Aspergillus fumigatus conidia	+	+	
Aspergillus fumigatus hyphae	+	+	

Fig. 16.31 Monocyte/macrophage killing of fungi. Many fungi are killed by monocytes or macrophages. Since cells from patients with chronic granulomatous disease and individuals with myeloperoxidase deficiency can also effect killing, this shows the importance of non-oxygen dependent mechanisms.

(see Chapter 22). Disturbance of normal physiology by immunosuppressive drugs or of normal flora by antibiotics can predispose to invasion by *Candida*. *Candida* infections are also common in immunodeficiency diseases (severe combined immunodeficiency, thymic aplasia, etc.) implying that the immune system is involved in confining the fungus to its normal commensal sites.

There is also evidence for neutrophil involvement in immunity to some respiratory mycoses such as mucormycosis (Fig. 16.30). It is likely that the cationic proteins discussed earlier in relation to bacteria are important for protection from fungi, since cells from patients with defective oxygen reduction pathways usually kill yeast and hyphae with near normal efficiency (Fig. 16.31). As with bacterial infections different mechanisms are active against different organisms.

VACCINE DESIGN

In order to design a vaccine, the following knowledge is required.

1. The relevant protective antigen(s).
2. The anatomical site where the mechanism needs to be expressed.
3. The immunological mechanism required.
4. An adjuvant and immunization schedule which is safe to use and will evoke the relevant response in that site.

All four criteria are fulfilled by those organisms which owe their pathogenicity to a single identified immunogenic protein. Toxins such as those of *Corynebacterium diphtheriae* and *Clostridium tetani* lose their toxicity when heated, but retain their immunogenicity. Such a toxoid will evoke a systemic antibody response when injected with a simple adjuvant such as $Al(OH)_3$ or killed *Bordetella pertussis*. Thus adjuvants for systemic antibody responses are not a problem.

Relevant Antigens Organisms such as *Streptococcus pyogenes* and *Str. pneumoniae* have large numbers of serotypes, so that an effective vaccine becomes a complex mixture which is expensive to make and incomplete in its coverage. Problems are still greater when there are numerous toxic products, but there is little definitive information as to which ones are protective antigens, or even the mechanism of protection. *B. pertussis* is an example, where the efficacy of a crude killed vaccine is due to luck rather than science. Neither its action, nor its occasional association with brain damage are understood, so a truly rational and safe vaccine cannot yet be designed.

Localization of Effect The next type of problem is the need to achieve expression of immunity in certain sites such as the genito-urinary tract or gut. Experimentally direct intravaginal immunization with antigens of *Neisseria gonorrhoeae* is much more effective than systemic immunization at evoking a response in this site. Similarly the conventional *Vibrio cholerae* vaccine injected intramuscularly has a very limited protective effect, while experimental oral vaccines can be more effective. The answer probably lies in the development of stable mutants of pathogenic organisms which after ingestion initiate an infection, and invade the local epithelium or lymphoid tissue, but die after a limited number of replication cycles. Thus derivatives of *Salmonella typhimurium* have been constructed which carry stable mutations determining the period of survival in the gut-associated lymphoid tissue and spleen. Such mutations can be transferred to other species, or the genes for relevant antigenic determinants from other pathogens can be inserted into the mutant, in the hope that they will be expressed in a way which will evoke appropriate responses.

Immunological Mechanisms When the required mechanism of response is cell-mediated, there are two problems for the designer of vaccines. First, for bacteria such as *Mycobacterium leprae* or *M. tuberculosis* protective antigens have not been identified. Secondly, when an adjuvant is required which is acceptable for use in man and will evoke a cell-mediated response rather

Corynebacterium diphtheriae *Clostridium tetani*	toxin	neutralizing antibody	$Al(OH)_3$ or pertussis	systemic
Streptococcus pneumoniae	capsular polysaccharide but many serotypes	antibody		systemic
Bordetella pertussis	not certain various toxins	? antibody		systemic + secretory
Neisseria gonorrhoeae	pili, LPS	antibody	? recombinant commensal	GU tract
Vibrio cholerae	toxin, LPS	antibody	? recombinant organism	gut
Mycobacterium tuberculosis	not known	? T cell-dependent macrophage activation	BCG - but often fails, and is a live vaccine	systemic

Fig. 16.32 Requirements for vaccine design. Different organisms require different strategies for vaccine design. In general those further down this list present increasing problems.

than only antibody, there are a few alternatives to live BCG (Bacille Calmette Guérin) – an attenuated strain of *M. bovis* – though the orally ingested *Salmonella* mutants discussed above may have some ability to evoke cell-mediated immunity (CMI) and vaccinia virus has also been considered. Therefore even if we could identify and clone the genes for a manageable number of protective antigens, they would probably need to be expressed in BCG before they could be used to evoke CMI. The technology for doing this has now been developed and BCG derivatives expressing protective epitopes from, for instance, *Leishmania* species are an exciting prospect.

Adjuvants Attempts are being made to develop safe adjuvants derived from the concept of Complete Freund's Adjuvant. This is a water-in-oil emulsion containing killed mycobacteria, which has severe side effects in humans. It is possible that isolated or synthetic adjuvant-active components of bacteria, such as derivatives of muramyl dipeptide, in a metabolizable oil, such as squalene, will prove acceptable in man.

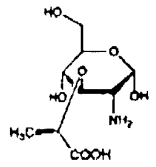
Ultimately we will need adjuvants for CMI which can direct the response preferentially towards particular subsets of cell-mediated mechanisms, for example cytotoxic T cells versus T cells mediating delayed hypersensitivity. At present we have no idea how to do this, though in some virus models activation of the wrong T cell subset can increase rather than decrease susceptibility to the infection. The problems associated with vaccine design are illustrated in Fig. 16.32.

Crystals from ether, mp 77-78° $[\alpha]_D^{20}$ −19.3° (c = 1 in methanol). uv max (ethanol): 222 nm (ε 14500).

THERAP CAT: Topical antibacterial.

THERAP CAT (VET): Topical antibacterial.

6384. Muramic Acid. *(R)-2-Amino-3-O-(1-carboxyethyl)-2-deoxy-D-glucose; 3-O-α-carboxyethyl-D-glucosamine.* $C_9H_{17}NO_8$; mol wt 251.24. C 43.03%, H 6.82%, N 5.58%, O 44.58%. Amino sugar found (as the *N*-acetyl derivative) in *peptidoglycan*, the main skeletal component of the bacterial cell wall. Discovery: J. T. Park, *J. Biol. Chem.* **194**, 885 (1952). Isoln from spores of *Bacillus megatherium*: R. E. Strange, F. A. Dark, *Nature* **177**, 186 (1956). Identification and synthesis: R. E. Strange, L. H. Kent, *Biochem. J.* **71**, 333 (1959). Stereospecific synthesis: Y. Matsushima, J. T. Park, *J. Org. Chem.* **27**, 3581 (1962); *eidem, Biochem. Prepn.* **10**, 109 (1963); T. Osawa, R. W. Jeanloz, *J. Org. Chem.* **30**, 448 (1965). Review of peptidoglycan structure: H. J. Rogers, *Ann. N.Y. Acad. Sci.* **235**, 29-51 (1974). Use to determine bacterial levels in mammalian tissues: J. Gilbart *et al., J. Microbiol. Methods* **5**, 271 (1986); in airborne dust: A. Fox *et al., Appl. Environ. Microbiol.* **59**, 4354 (1993).

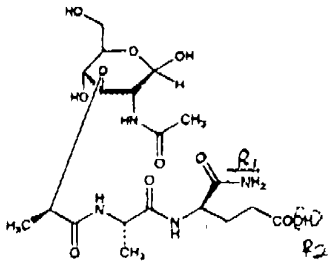


Crystals from water, mp 152-154° (dec): $[\alpha]_D^{25}$ +103° (c = 0.26 in water) (Matsushima, Park). Also reported as crystals from 90% ethanol, mp 160-162° (dec): $[\alpha]_D^{22}$ +146° (6 minutes) → +116° (31 hrs) (c = 0.57 in water) (Osawa, Jeanloz).

N-Acetylmuramic acid. $C_{11}H_{19}NO_8$. *(R)-2-(acetylamino)-3-O-(1-carboxyethyl)-2-deoxy-D-glucose.* Crystals from ethyl acetate + methanol, mp 119-121° $[\alpha]_D^{22}$ -56° (10 minutes) → +40° (24 hrs) (c = 0.68 in water).

USE: As chemical marker for the detection of bacterial contamination.

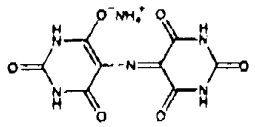
6385. Muramyl Dipeptide. *N²-[N-(N-Acetylmuramoyl)-L-alanyl]-D-α-glutamine; N-acetylmuramyl-L-alanyl-D-isoglutamine; 2-acetamido-2-deoxy-3-O-(D-2-propionyl-L-alanyl-D-isoglutamine)-D-glucopyranose; MDP.* $C_{19}H_{32}N_4O_{11}$; mol wt 492.48. C 46.34%, H 6.55%, N 11.38%, O 35.74%. Synthetic immunoadjuvant corresponding to the smallest immunologically active glycopeptide subunit of the bacterial cell wall. Prepn: A. Adam *et al.*, Ger. pat. **2,450,355**; *eidem*, U.S. pat. **4,235,771** (1975, 1980 both to Agen. Nat. Valor. Recher.); C. Merser *et al., Biochem. Biophys. Res. Commun.* **66**, 1316 (1975); P. Lefrancier *et al., Int. J. Pep. Prot. Res.* **9**, 249 (1977). Structure-activity relationships: F. Ellouz *et al., Biochem. Biophys. Res. Commun.* **59**, 1317 (1974); A. Adam *et al., ibid.* **72**, 339 (1976). Review of immunoregulating activity: L. Chedid *et al., Prog. Allergy* **25**, 63-105 (1978). Tissue distribution in mice: M. Parant *et al., Int. J. Immunopharmacol.* **1**, 35 (1979). Potentiation of antitumor immunity: S. Sone *et al., J. Biol. Resp. Mo* 185 (1984); A. E. Eggers, *ibid.* **7**, 229 (1988).



Crystals from methanol-acetone-ether. $[\alpha]_D^{25}$ +44° (c acid).

USE: Immunological adjuvant.

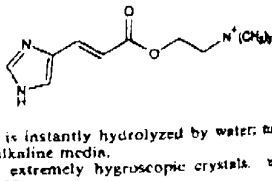
6386. Murexide. *5-[(Hexahydro-2,4,6-trioxo-5-pyr inyl)amino]-2,4,6(1H,3H,5H)-pyrimidinetrione monoa nium salt; 5,5'-nitrilodibarbituric acid monoammoniu* acid ammonium purpurate; ammonium purpurate. N_8O_6; mol wt 284.19. C 33.81%, H 2.84%, N 29.57 33.78%. Prepn from alloxan + NH_3: Hartley, *J. Chem* **87**, 1791 (1905); Schwartz, Handritschk, East Ger. pat 589 (1959), *C.A.* **55**, 3630a (1961); from alloxantin + a nium acetate: Davidson, *J. Am. Chem. Soc.* **58**, 1821 (Structure: Hartley, *J. Chem. Soc.* **87**, 1796 (1905); Wi *J. Am. Chem. Soc.* **61**, 2089 (1939); Schreiber, *Mitt Pharm. Ges.* **28**, 20 (1958).



Purple-red crystals with green metallic luster. Ab tion max in water: 520 nm. Sparingly sol in cold more in hot water; practically insol in alcohol, ether. H_2O soln is deep purple and the aq NaOH soln is deep

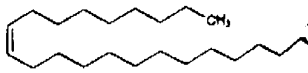
USE: Indicator for complexometric titrations.

6387. Murexine. *2-[[3-(1H-Imidazol-4-yl)-1-oxo penyl]oxy]-N,N,N-trimethylethanaminium; β-(4-imida acrylcholine; urocanylcholine.* $[C_{11}H_{19}N_3O_2]^+$. Nepr ocular blocker found, often in very large quantities, median zone of the hypobranchial body of *Murex tr* and of other related species of mollusks: Erspamer, doni, *Ric. Sci. Ricostr.* **16**, 1114 (1946); *Arch. Int. Ph codyn.* **74**, 263 (1947); Erspamer, *ibid.* **76**, 308 (1948). *idem, Experientia* **4**, 226 (1948); M. Roseghini *et al., Biochem.* **12**, 468 (1970); J. E. Blankenship *et al., Biochem. Physiol.* **51C**, 129 (1975). Synthesis: Pasini *Ann.* **578**, 6 (1952); Pasini, Coda, U.S. pat. **2,956,063** to Società Farmaceutici). Structure: V. Erspamer, G ti, *Science* **117**, 161 (1953). Effect on vertebrate and tebrate muscles: J. C. de Freitas, *Comp. Biochem. P* **56C**, 57 (1977).



The base is instantly hydrolyzed by water; unstab in acid or alkaline media.

Chloride, extremely hygroscopic crystals. uv m 4.5): 280-282 nm.

Chloride hydrochloride, hygroscopic microcry powder, mp 219-221° (dec); shows the same uv max chloride.

6388. Muscalure. *(Z)-9-Tricosene; cis-tricos* $C_{23}H_{46}$; mol wt 322.62. C 85.63%, H 14.37%. Sol mone of the female common house fly, *Musca dom* Isoln and synthesis: Carlson *et al., Science* **174**, 76 Alternate syntheses: Eiter, *Naturwiss.* **59**, 468 (197 gill, Rosenblum, *J. Org. Chem.* **37**, 3971 (1972); *et al., Chem. Commun.* 1973, 735; Ho, Wong, *Can. J.* **52**, 1923 (1974); K. Abe *et al., Bull. Chem. Soc. Jp* 2792 (1977); V. N. Odinokov *et al., Tetrahedron Le* 1371 (1982).